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High temperature aggravates the effects of pesticides in goldfish

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ABSTRACT

In human-altered rivers, fish are often conjointly exposed to an increase in water temperature due to global warming and to a contamination by organic pollutants such as pesticides, but their combined effects are still elusive. Thermal and chemical stressors could potentially interact because high temperature increases metabolism and toxicant uptake, and can alter the ability of organisms to set up adequate stress responses and to maintain homeostasis. These combined stressors could thus potentially result in higher level of molecular and cellular damage, and stronger effects on behavior and physiology, but experimental evidence across biological levels is still scarce. In this study, goldfish *Carassius auratus* were experimentally exposed to an environmentally realistic cocktail of pesticides (S-metolachlor, isoproturon, linuron, atrazine-desethyl, aclonifen, pendimethalin and tebuconazol) commonly found in rivers of South-West of France at low or high dose in two different thermal conditions: a common summer temperature (22 °C) or a high temperature recorded during heat waves (32 °C). Results showed that high temperature alone caused behavioral and physiological changes (increased swimming activity, increased hepatosomatic index, decreased reproductive index) but limited cellular damage. However, high temperature aggravated the effects of pesticides at the molecular and cellular level. Indeed, pesticide exposure resulted in higher genotoxic effects (micronuclei rate) and irreversible cellular damage of the gills and liver (apoptosis, inflammation, necrosis) at 32 °C compared to 22 °C. This suggests potential synergistic effects of climate change and pollution, and highlights the need for multiple stress approaches to better predict the impacts of human activities on aquatic wildlife.

1. Introduction

In the context of global change, aquatic organisms are exposed to a growing number of environmental stressors that create new selective pressures affecting their behavior, physiology and ultimately their fitness (Crozier and Hutchings, 2014; Schinegger et al., 2018; Schulte, 2014). Although a considerable body of literature addressed the isolated effects of different stressors on aquatic organisms, few studies investigated their combined effects, thereby hindering our ability to predict the effects of human-induced perturbations in realistic environmental conditions (Holmstrup et al., 2010; Matthaei and Lange, 2016; Nõges et al., 2016; Schinegger et al., 2018, 2016). In human-altered rivers, fish are often conjointly exposed to an increase in water temperature due to global warming, especially during heat waves, and to a concomitant contamination by organic pollutants such as pesticides, which are widely used in urban and agricultural areas (Page et al., 2014; Polard et al., 2011a). In most cases, rivers were contaminated by complex mixtures of pollutants, including several pesticides, which can induce different effects (Hayes et al., 2006; Relyea, 2009; Wang et al., 2017). In addition, thermal and chemical stressors can potentially interact and alter the ability of organisms to set up adequate defensive stress responses and to maintain homeostasis (Gandar et al., 2017a; Nadal et al., 2015; Noyes et al., 2009; Schiedek et al., 2007). Indeed, high temperatures can directly increase the intake and toxicity of organic molecules (Laetz et al., 2014; Nadal et al., 2015). In addition, high temperature triggers costly stress responses, accelerate metabolism and deplete energy reserves, which could indirectly affect ability of organisms to set up efficient defense responses against

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chemicals and to limit cellular damage (Gandar et al., 2017a, 2015; Kennedy and Ross, 2011). Increased temperature can be particularly deleterious for ectothermic species as temperature affects most of their behavioral and metabolic processes (Manciocco et al., 2014). As a consequence, thermal stress might amplify or mask the effects of toxic stressors in fish, making it difficult to predict their combined effects at different levels of biological organization, from the cell to the whole organism.

To tackle this question, we used goldfish *Carassius auratus* as a model species because of its extensive use in ecotoxicological tests and for its responsiveness to chemical and natural stressors (Cavas, 2011; Gandar et al., 2017b; Maisano et al., 2013; Parrino et al., 2018). It is non-native species from Asia, that can now be found in a wide range of lentic and lotic systems across Europe, including in the Garonne watershed, South-West of France (Keith et al., 2011). Goldfish were experimentally exposed to an environmentally realistic cocktail of common pesticides (S-metolachlor, isoproturon, linuron, atrazine-desethyl, aclonifen, pendimethalin and tebuconazol) at low and high concentrations observed in rivers of South-West of France (Polard, 2010; Polard et al., 2011a). This experiment was conducted with a temperature of 22 °C, currently registered in rivers of South-West of France and corresponding to the optimal temperature for goldfish, and with a high temperature (32 °C), which is within the upper tolerance limit of goldfish (0.3–12.6 °C for the lower limit and 30.8–43.6 °C for the upper limit, Ford and Beitinger, 2005; Fry and Hart, 1948). The chosen high temperature of 32 °C is a temperature observed in the Garonne river (located in South-West France) during extreme weather events such as heat waves, which will increase in intensity and frequency in the future due to climate change (IPCC, 2013). Fish were exposed to a high temperature or pesticides alone or to both stressors in combination.

To understand better the effects of chemical and thermal stress, alone or together, the behavioral and physiological responses of fish were analyzed at different levels of biological organization. Pesticides generally induce pathological damage at the molecular, cellular and individual levels, depending on the stress intensity and the duration of exposure. At the molecular level, genotoxic effects of pesticides are often observed, with alterations of DNA structure that can result in the production of micronuclei and alter the cell cycle (Cavalcante et al., 2008; Polard et al., 2011a). Previous proteomic studies on *Carassius auratus* showed that the cocktail of pesticides used in this study affected proteins involved in cellular inflammatory responses, oxidative stress, and in cytoskeleton and cell structure (e.g. Gandar et al., 2017b; Marchand et al., 2006). However, the consequences at the cellular level are not known yet. Oxidative stress and proteome changes caused by pesticides are expected to result in apoptosis or accelerated cell cycle, tissue lesions and potentially carcinogenesis, especially in the liver (Gandar et al., 2017b; Ratn et al., 2017; Weber et al., 2013; Wirbisky et al., 2016). In this study, we thus focused on histopathological modifications of the gills and liver, because they are the two primary target organs affected by organic pollutants (e.g. Nero et al., 2006). Gills are an important interface between the external and internal environment and can be a route of contaminant uptake in fish (Evans, 1987). Liver is the primary organ for detoxification and proteomic studies showed differential levels of proteins involved in hepatocyte apoptosis and cirrhosis in goldfish exposed to pesticides (Gandar et al., 2017b).

Given previous proteomic studies (Gandar et al., 2017b), we hypothesized that pesticide exposure would alter liver and gill structure and result in cellular lesions (such as necrosis, inflammatory lesions), especially at high temperature. We also hypothesized that pesticides would reduce gonad mass (often measured by Gonadosomatic Index), liver mass (Hepatosomatic Index) and body condition, because stressful conditions often decrease investment in reproduction (Schreck, 2010) and lead to reallocation of energy reserves (Gandar et al., 2015). At the individual level, temperature and organic pollution also often trigger immune changes (here measured as changes in innate immunity) (Fatima et al., 2007) and behavioral changes (activity, feeding, exploration) (Gandar et al., 2015; Jacquin et al., 2017), although their combined effects are still elusive. Based on previous metabolomic approaches showing synergistic effects of temperature and pesticides on goldfish immune proteins, energy allocation and maintenance (Gandar et al., 2017b, 2017a, 2015), we predicted that pesticides would have stronger effects at 32 °C on immunity and behavior compared to 22 °C.

2. Materials and methods

2.1. Model species

The goldfish (*Carassius auratus*, Cyprinidae family) was chosen as a biological model for this study because of its extensive use in ecotoxicological tests (e.g. Bretaud *et al.*, 2002, Cavas 2011, Feng *et al.*, 2013). Introduced in Europe in the eighteenth century for ornamentation, it is a non-native species from Asia that can now be found in a wide range of lentic and lotic systems across Europe, including in the studied Garonne watershed, South West of France (Keith et al., 2011). The temperature tolerance of the goldfish is large (0.3–12.6 °C for the lower limit and 30.8–43.6 °C for the upper limit) and its optimum temperature is 25 °C. (Ford and Beitinger, 2005; Fry and Hart, 1948). A total of 156 individuals of 10–12 cm in length were purchased from the fish farm Carpio (Consac, France). They were acclimatized for two weeks in opaque tanks under controlled conditions (18 °C with a 12:12 h light regime). They were then gradually acclimatized to common (22 °C) or high (32 °C) experimental temperatures. The temperature was increased by 0.5 °C every 12 h until reaching the experimental temperature following previous studies (Gandar et al., 2015). Water was aerated and dechlorinated prior to fish introduction. Half of the water was renewed every day and fish were fed daily with commercial pellets (Carpio, France).

2.2. Experimental treatments

A total of 156 fish (26 fish in each experimental group) were placed in 30 L opaque aquaria (22 and 32 °C). Temperature regime was controlled using heaters coupled with thermostats. Temperature was monitored every 24 h to ensure constant conditions throughout the exposure period. The 22 °C temperature was chosen as a control temperature since it is close to goldfish optimal temperature range, while 32 °C corresponds to a stressful temperature within the upper tolerance limit of goldfish. The 22 °C temperature is a common summer temperature in rivers in South-West of France. The 32 °C temperature corresponds to a high river temperature recorded during heat waves (data from Agence de l'Eau Adour-Garonne, Croze et al., 2007), which are expected to increase in frequency and intensity in the next decades due to climate change (IPCC, 2013).

In each temperature group, fish were exposed either to control water (C), or to a low (L) or high dose (H) of a cocktail of pesticides during 96 h, resulting in 6 experimental groups of 26 fish (C22, L22, H22, C32, L32 and H32). The cocktail of pesticides contains seven most common pesticides: 6 herbicides (S-metolachlor, isoproturon, linuron, atrazine-desethyl, aclonifen and pendimethalin) and one fungicide (tebuconazol), which are mainly used for intensive agriculture (corn, wheat and sunflower) (details in Table 1). This pesticide composition and concentrations were selected according to preexisting data of a typical pesticide contamination in the rivers of the South-West of France (Polard, 2010; Polard et al., 2011a; Taghavi et al., 2011). Two concentrations were chosen: a low dose of 8.4 $\mu g\,L^{-1}$ (L groups) and a high dose of 42 $\mu g\,L^{-1}$ (H groups). The Low Dose corresponds to a typical pesticide concentration found after spring flood in French rivers (Debenest et al., 2010; Garmouma et al., 1998; Polard et al., 2011a; Taghavi et al., 2011). For instance Polard et al. (2010, 2011a), Taghavi et al. (2011) and other authors found approximately 2,0 to 2,4 µg/L of metolachlore, 1,0 to 1,4 µg/L of isoproturon and related molecules, and

Table 1
Composition of the pesticide cocktail, based on environmental measures from French agricultural rivers (see methods).

Chemicals	Family	Use	Action mode	Low Dose µg/L	High Dose µg/L
S-metolachlor	Chloroacetanilide	Herbicide	*Mitosis perturbation*	2.4	12.0
Linuron	Urea	Herbicide	*Photosystem II inhibitor*	2.0	10.0
Isoproturon	Sustituted urea	Herbicide	*Photosystem II inhibitor*	1.2	6.0
Tebuconazole	Triazole	Fungicide	*Sterol synthesis inhibitor*	1.2	6.0
Aclonifen	Diphenyl ether	Herbicide	*Carotenoid inhibitor*	0.8	4.0
Atrazin-desethyl	Triazine	Herbicide	*Photosystem II inhibitor*	0.4	2.0
Pendimethalin	Dinitroaniline	Herbicide	*Mitosis perturbation*	0.4	2.0
Total				**8.4 µg/L**	**42 µg/L**

0,2 to 0,4 µg/L of atrazine and related molecules in the Save river after spring floods. Data from the French national database on water pollution show that these concentrations are in the range of what can be found in other agricultural French rivers (IFEN 2007). The high concentration (H: High dose of 42 µg L^{-1}) is five times higher and reflects highest levels of contamination measured in streams and reservoirs of Europe and USA (Battaglin et al., 2000; Graymore et al., 2001; Kreuger, 1998). Details of these concentrations for each pesticide are in Table 1.

Pesticides were obtained from Sigma-Aldrich (St. Louis, MO, USA): S-metolachlor (CAS-No: 87392-12-9, PESTANAL®, analytical standard), Isoproturon (CAS-No: 34123-59-6, PESTANAL®, analytical standard), Linuron (CAS-No: 330-55-2, PESTANAL®, analytical standard), Atrazine-desethyl (CAS-No: 6190-65-4, PESTANAL®, analytical standard), Aclonifen (CAS-No: 74070-46-5, PESTANAL®, analytical standard), Pendimethalin (CAS-No: 4048742-1, PROWL®, analytical standard), Tebuconazol (CAS-No: 107534-96-3, PESTANAL®, analytical standard). Pesticides were diluted in acetone (0.3 mL L^{-1} of water) and demineralized water to obtain the two concentrations selected. Acetone (CAS: 67-64-1, Fisher Chemical, HPLC solvent) was purchased from Fisher Scientific (Illkirch, France). Control groups (C22 and C32) received only acetone. The composition of the mixture, characteristics and concentrations of pesticides are presented in Table 1.

Prior to fish introduction, aquaria and all equipment were pre-saturated with the mixture of pesticides. Air pumps were placed in each aquarium to supply oxygen in water. Half of water and pesticide solutions was renewed every day to limit concentration variations. Previous studies showed that this protocol successfully maintained pesticide concentrations in the water and that good oxygen and pH conditions across the experiment (Gandar et al., 2015, 2017). No mortality occurred during the exposure. At the beginning of the experiment at T0, groups did not differ in body mass (χ_2 = 1.51, p = 0.91). At the end of the experiment and after the behavioral tests, fish were weighed (to the nearest 10 mg) and measured (fork length to the nearest mm). Fish were anesthetized and euthanized with benzocaine (80–120 mg L^{-1}) and concussion.

2.3. Molecular level: DNA alterations

DNA alterations were estimated on blood smears by counting the Micronuclei (MN) rate in erythrocytes following the protocol described in Polard et al. (2011b) on half of the individuals of each treatment group (N = 12–14 randomly chosen slides per group). Micronuclei rate is used to quantify the genotoxicity of treatments, which might arise through mutagenic of epigenetic processes (Al-Sabti and Metcalfe, 1995; Luzhna et al., 2013; Torres de Lemos et al., 2007). Blood was sampled in the caudal vein using 1 mL Terumo syringe with a 26 G needle and one drop was fixed on slides. Blood slides were fixed in pure ethanol for 15 min and dried for 24 h. All slides were then stained with fluorescent acridine orange AO (0.003% in Dulbecco PBS) (Polard et al., 2011b). Micronuclei yellow-green fluorescence was counted under epifluorescence with a U-MWB2 filter at 1000 magnification. Micronuclei rates were calculated as the number of micronuclei per 5000 blood cells.

2.4. Cellular level: histopathological alterations of the gills and liver

Histopathological lesions were evaluated in the liver (26 fish per experimental group, 3 observations per individual) and in the gills (3 females per experimental group, 30 observations per individual, 10 measurements per filament).

Gill and liver damage was assessed using standard histological techniques (Bernet et al., 1999; Costa et al., 2011; Cuevas et al., 2015; van Dyk et al., 2007). Organs were dissected and immediately fixed in Bouin's fluid (picric acid 3 V, formaldehyde 1 V and acetic acid 2 V) for 24 h. They were dehydrated through a graded ethanol series (70–100%), following by Butanol-1ol bath, cleared in toluene, and embedded in paraffin. Longitudinal serial sections of 10 µm (gills) or 6 µm (liver) were mounted on glass slides, stained with hematoxylin-eosin HE (gills) or with nuclear fast red NR and picro-indigo-carmin PIC (liver), and observed under Nikon microscope (10 ×, 40X and 60 ×). Microphotographies were taken with a light microscope (Laborlux S – Leitz wetzlar Germany) equipped with a digital camera (Imagingsource) and analyzed using Archimed software (Version 5.6.0.).

Gill remodelling was assessed using the percentage of exchange surface area (ESA), corresponding to protruding length of secondary lamellae (10 measurements per filament in the middle of the arch, i.e. 30 measurements per sample, hence, 90 measures per conditions) and was calculated as: ESA (%) = 100 * (mean SL - mean ILCM) / mean SL) where: SL = length of secondary lamellae; ILCM = length of interlamellar cell mass. In addition, five filaments located in the middle of the arch (i.e. 5 measures per fish, namely 15 measures per condition) were analyzed following Bernet et al. (1999) to calculate the degree of gill damage. Six different types of alterations were recorded (details in Table S1). A gill pathological index (I_{Gill}) was calculated as: $I_{Gill} = \Sigma_{alt}$ (a_{alt} x w_{alt}) where: a = score value (depending on occurrence) for a specific alteration (i.e. type of lesion), ranging from 0 to 6; w = importance factor for a specific alteration (1: reversible lesions with low effects; 2. modified cellular architecture: 3: degenerative irreversible alterations leading to a loss of gill function),; alt = specific alteration (see Table S1).

Six main hepatic alterations were recorded in the liver (on 3 random areas of 25 mm^2): lysis-necrosis, mitosis, melano-macrophage aggregates, fibrosis, nuclear alterations, and lipidosis. A global histopathological score was calculated as the mean value of the different scores (details in Table S1).

2.5. Organ level: GSI, HSI and body condition

Sex and maturity were determined by gonadal examination. The Hepatosomatic Index HSI representing the energy content of the liver and the Gonadosomatic Index GSI representing the gonad growth status were calculated as liver or gonad mass/total body mass. Body condition was measured by the Fulton condition index as (body mass/ body $length^3$).

2.6. Individual level: immunity

After centrifugation (10 min at 3000 rpm), plasma was frozen for subsequent immune analyses. Innate immunity was assessed by measuring lysozyme activity (which reflects the ability of the individual to eliminate bacteria), and peroxidase activity of the plasma (which is used as a marker of leucocyte activation, Eslamloo et al., 2014; Fatima et al., 2007; Ghafoori et al., 2014; Magnadóttir, 2006). To measure lysozyme activity, 8 mg of *Micrococcus lysodeikticus* were suspended in 40 mL of Na_2HPO_4 buffer/ 50 mM of citric acid (pH = 5.5). A total of 15 µL of plasma were placed in 96 wells microplates, with 150 µL of revealing solution. Optical density was read at 450 nm from 5 to 60 min. The absorbance was measured at 450 nm using a spectrophotometer at an interval of 30 s (total measuring time 60 min). The unit of lysozyme activity is defined as the concentration of sample causing a decrease in turbidity of 0.001/ min (1 UA = Δ 0.001 A_{450}/ min).

Peroxidase activity was measured following protocols from Quade and Roth (1997). Tetramethylbenzidine (TMB) and hydrogen peroxyde H_2O_2 were used as enzymatic substrates. A total of 12.5 µL of plasma was diluted in 0.5 mL of Hank buffer (HBSS). A total of 60 µL of this dilution were placed in a 96-microplate with a 100 µL solution of TMB, DMSO, Na_2HPO_4/citric acid 50 mM (pH = 5.0) buffer. After 2 min of incubation, 50 µL of 2 M sulfuric acid were added to stop the reaction. The absorbance was measured at 450 nm using a spectrophotometer, and expressed as UA with: UA = A_{450} "sample" - A_{450} "control" and used as a measure of peroxidase activity.

Because immunity can differ among individuals before the experiment depending on past exposure of fish to disease, we also measured their lysozyme and peroxidase levels in the plasma before the experiment to control for individual immune differences before experimental treatments. Lysozyme or peroxidase variations across the experiment were then calculated as (value after treatment-value before treatment)/ value before treatment.

2.7. Individual level: behavior

We measured activity, mean speed, trajectory changes, exploration and feeding behavior at T96 (96 h post pesticide exposure). These behavioral traits were chosen because they are often affected by stressors and are tightly linked to fish fitness and functional effects (e.g. Scott and Sloman, 2004, Gandar et al., 2015). Fish were not fed during the exposure to pesticides to standardize their physiological state and appetite.

First, we recorded the “activity” of fish in the home tank as the total time spent swimming during 5 min (i.e., in directed motion and covering a distance > 10% of body length). Activity was recorded by direct observation in the home tank with a companion fish to limit isolation stress and reliably reflects the intrinsic activity of the fish. Time of the day of observation was randomized for each group to avoid any confounding effect of diurnal rhythm. Second, we recorded more specific features of the trajectory of the fish such as “mean speed”, “trajectory changes”, and “exploration”, because contaminants can have neurotoxic effects that can result in erratic behaviours, affect trajectories and exploration patterns (e.g. Jacquin et al., 2017). For this, we transferred each individual in a test tank (50 × 30 cm with 10 cm of water) for 10 min with the same conditions as the exposure tank. Each focal fish was placed in a transparent cylinder in the middle of the tank. After 2 min of acclimation the cylinder was gently removed, using a string on a reel to minimize disturbance. Movements were then recorded precisely for 5 min using a camera (Logitech HD Webcam C270) the VirtualDub software and the Tracker software (Tracker 5.0, Open Source Physics 2015) to obtain precise coordinates of the position of the fish at each time point (x, y). This allowed us to obtain the mean speed of the fish (cm/s). The number of trajectory changes was also recorded as the number of rapid (in less than 5 s) and abrupt (180° and > 100% of body length) changes of direction.

Lines were drawn on the bottom of the tank to delimit 15 zones of 10 × 10 cm. Exploration was recorded as the number of different zones reached by the fish during 5 min (Jacquin et al., 2017). The position of the different test tanks was changed every 5 fish to avoid any confounding effect of a spatial preference. At the end of the behavioral test, we introduced 10 food pellets in the test tank and recorded the number of pellets consumed during one minute and the latency before feeding to compare the feeding behavior between groups.

2.8. Compliance with ethical standards

All experimental procedures were conducted in compliance with French and European legislation for animal experimentation (European directive 2010/63/UE). Experimental procedures were conducted under the French animal handler's certificate (No. 31-103), the establishment approval for vertebrate experimentation No. A3113002, and were approved by the ethical committee 073 (authorization No. 8538).

2.9. Statistical analyses

Effects of stressors (temperature or pesticides or both) were evaluated by Generalized Linear Models for parametric data with temperature, pesticide dose, and their interaction as explaining factors. Sex was also added in the initial models but was not retained in any model (all p values > 0.12), except for Gonadosomatic Index where separate analyses were conducted on males and females. Best models were chosen using a backward selection and successively dropping non-significant terms (p > 0.10). Posthoc tests were conducted using Wilcoxon tests. For non-parametric data, Kruskall-Wallis tests were conducted to compare groups. For feeding behavior, an additional analysis combining feeding latency and feeding status (at least one pellet eaten or no pellet eaten) was analyzed using survival curve differences (survdiff function, Kaplan-Meier method).

3. Results

3.1. DNA alterations and micronuclei

Micronuclei rate significantly differed between experimental groups (Kruskall-Wallis test: Chisq = 14.9, P = 0.011). Fish at 22 °C exposed to a high dose of pesticides had a marginally higher micronuclei rate than the control group (C22 vs H22: Wilcoxon test: W = 47, P = 0.057) (Fig. 1). Fish at 32 °C exposed to a high dose of pesticides had a significantly higher rate of micronuclei compared to the C22 control group (Wilcoxon test, C22 vs H32: W = 47, P = 0.029) and marginally higher compared to the C32 group (H32 vs C32: W = 56, P = 0.094) (Fig. 1).

3.2. Histopathological alterations of the gills

Histopathological alterations of the gills significantly differed between groups (details in Table S2). Fig. 2 shows the different types of changes and lesions observed in the gills of exposed fish. At 22 °C in the control C22 group, no tissue injuries were observed and the lamellae were embedded in a thick ILCM (C22, Fig. 2), resulting in a low Exchange Surface Area (ESA = 5.0 ± 11.5; Table S2). At 22 °C with pesticides in the L22 group, ILCM length decreased, and partial fusion of secondary lamellae (Fu) and abundant mucus occurred (L22, Fig. 2). In the H22 group, the ILCM was very reduced (H22, Fig. 2), resulting in a significant increase of exchange surface area ESA, that reached 82% for H22 fish (Table S2).

At high temperature in the C32 group, the elevation of temperature to 32 °C also induced a decrease of ILCM in C32 fish (C32, Fig. 2) resulting in a significant increase of exchange surface area ESA (77.5 ± 4.4% in C32 versus 5.0 ± 11.5% in C22, Table S2), and protective mucus was present. Protective mucus was absent in fish

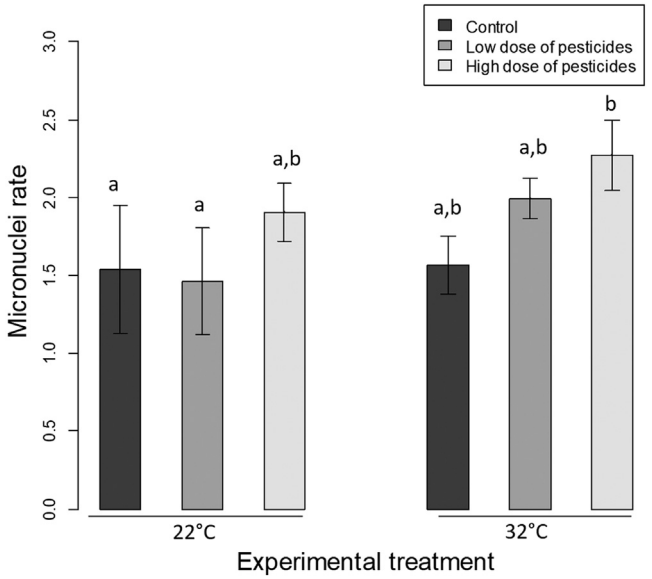

Fig. 1. Level of DNA damage estimated by micronuclei rate (mean ± SE) after a 96 h exposure at 22 °C (left) or 32 °C (right) to control water (dark grey), a low dose of pesticides (medium grey), or a high dose of pesticide (light grey) Different letters above bars indicate significant differences between groups ($P < 0.05$).

exposed both to 32 °C and to pesticide mixtures (L32, H32 Fig. 2). Lamellar hyperplasia (Hy) was present and pesticides induced lifting of lamellar epithelium (Li), and oedema (Oe) in H22 fish (Fig. 2; Table S2). At 32 °C with high dose of pesticides (H32), irreversible lesions appearing frequently, especially necrosis (Ne), and oedema (Oe) (Fig. 2; Table S2).

As a result, the integrative gill pathological index (I_{Gill}) thus increased in 32 °C group compared to 22 °C group and in fish exposed to a high dose of pesticides at 22 °C and 32 °C (Fig. 3A). Fish exposed to both stressors (H32) exhibited a higher level of gill pathological index as compared to all other groups of fish, reflecting a highly degraded cellular structure of the gills (Fig. 3A).

3.3. Histopathological alterations of the liver

High temperature alone had no significant effect on fish hepatic structure (C32 vs C22) (Fig. 3B). In contrast, exposure to pesticides induced fibrosis, an increase of the immune response and hepatocyte nuclear alterations typical of an inflammatory syndrome (details in Supplementary Fig. S3 and S4), resulting in a higher histopathological score in L22 and H22 fish compared to C22 (Fig. 3B). At high temperature, most fish exposed to pesticides (L32 and H32) showed disorganization of some hepatic areas, i.e. an increase in the number of hepatocytes involved in mitosis and inflammation, lipid droplets and a proliferation of blood vessels (details in Supplementary Fig. S4). A total of 80% of fish exposed to the high concentration of pesticides and to 32 °C (H32) had a significant inflammatory response in the liver (Fig S4). The global histopathologic index shows that perturbation of the liver due to pesticides was more severe at 32 °C compared to 22 °C (Fig. 3B).

3.4. HSI, GSI, body mass loss

The Hepatosomatic Index (HSI) was affected by temperature only (Table 2), with fish exposed to 32 °C having a higher HSI than fish exposed to 22 °C ($t = 2.10$, $P = 0.042$). The Gonadosomatic Index (GSI) of females (but not in males) was significantly affected by temperature

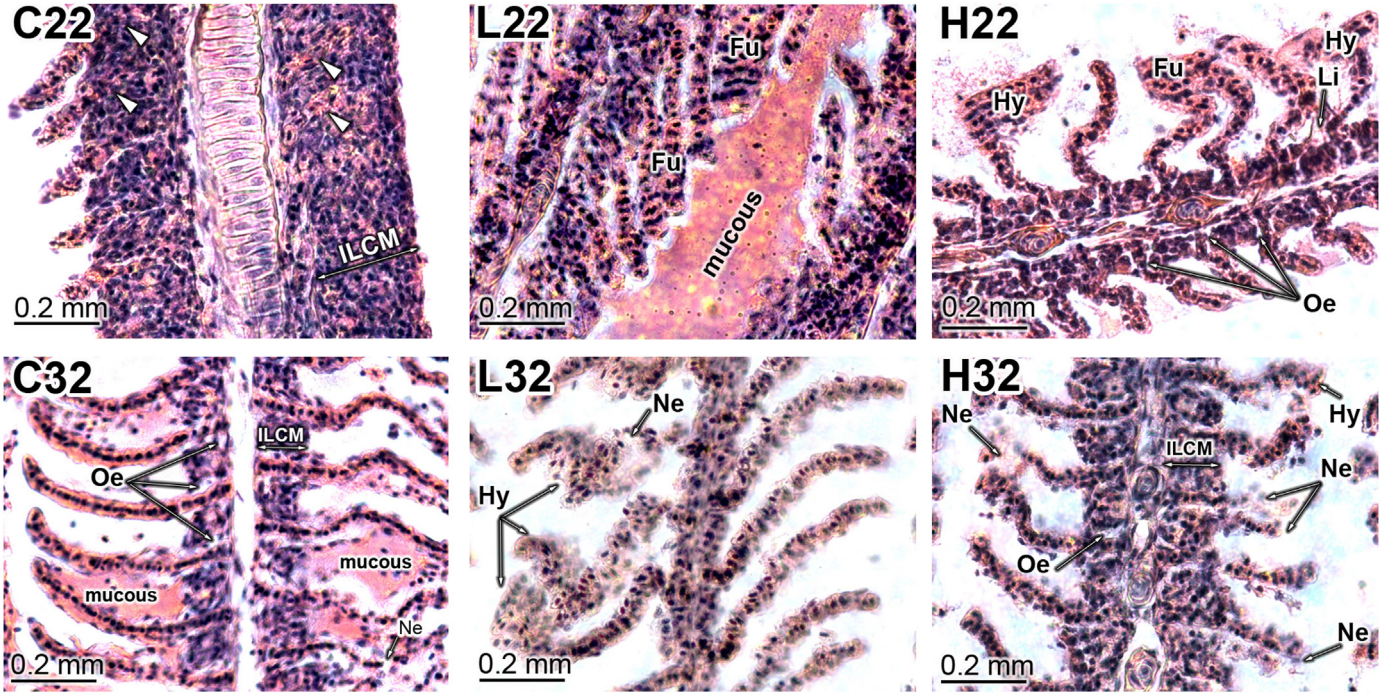

Fig. 2. Gill histological longitudinal sections (**C22**) Control at 22 °C. White arrowheads indicate secondary lamellae embedded in thick InterLamellar Cell Mass (ILCM, white double arrow) in control fish. (**L22**) Low Dose of pesticides at 22 °C. Mucus is present, and ILCM between the gill filaments are reduced and lesions such as fusions of secondary lamellae (Fu) appear. (**H22**) High Dose at 22 °C. More types of lesions are observable, including Oedema (Oe). (**C32**) Control at 32 °C. The ILCM is very reduced, increasing the Exchange Surface Area (ESA). (**L32**) Low Dose of pesticides at 32 °C. Hyperplasia (Hy) and irreversible lesions such as necrosis (Ne) occur. (**H32**) High Dose of pesticides at 32 °C. Secondary lamellae are shorter and most types of lesions are present and frequent: necrosis (Ne) is abundant and all gill filaments show severe tissue lesions. Fu: partial fusion of secondary lamellae; Hy: lamellar Hyperplasia; ILCM: interlamellar cell mass, Li: Lifting of lamellar epithelium, Ne: Necrosis, Oe: intercellular Oedema.

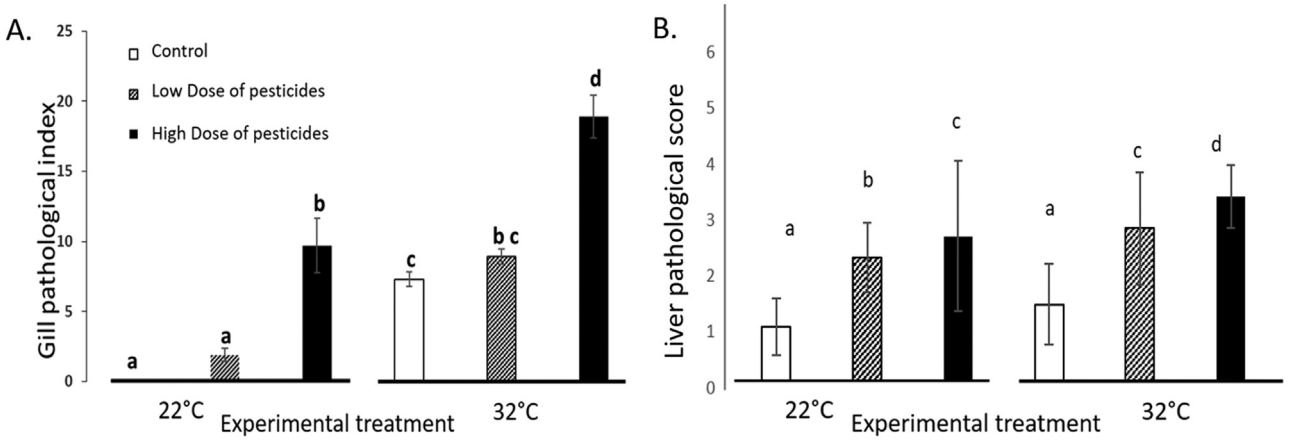

Fig. 3. A. Gill pathological index ($I_{Gill,}$ mean ± SE) and B. global score of histopathologic alterations (mean ± SE) in Carassius auratus exposed during 96 h to pesticides at different concentrations (Control in white, Low Dose in grey, and High Dose in black) and at two temperatures (22 °C on the left and 32 °C on the right). A high index indicates a high degree of cellular damage. Data are mean ± SEM. Different letters above bars indicate significant differences between groups.

Table 2
Best Generalized Linear Models (GLM) explaining physiological and behavioral traits in goldfish exposed to temperature increase and/or pesticides. Sex was included as explanatory variable but was not retained in any model (see results). Separate models on males and females were run for GSI. Non parametric analyses were run on other traits and presented in the text.

	Temperature		Pesticides		Temp*Pesticides	
	LR χ_2	P	LR χ_2	P	LR χ_2	P
Organs and body condition						
Hepatosomatix Index HSI	4.41	0.036	–	–	–	–
Male Gonadosomatic Index GSI	–	–	–	–	–	–
Female Gonadosomatic Index GSI	0.30	0.58	9.72	0.0078	9.46	0.0088
Body mass loss	6.91	0.0086	–	–	–	–
Immunity						
Lysozyme activity	–	–	–	–	–	–
Peroxidase activity	0.0002	0.98	5.68	0.058	6.67	0.035
Behavior						
Activity	38.31	< 0.001	10.47	0.0053	–	–
Speed	4.00	0.045	–	–	–	–
Trajectory changes	5.86	0.015	–	–	–	–
Exploration	9.29	0.0022	–	–	–	–
Feeding	5.84	0.016	9.27	0.0097	–	–

and pesticides (Table 2). More precisely, at 22 °C, pesticides decreased female GSI at low dose (W = 136, P = 0.026), and marginally at high dose (W = 108, P = 0.10), whereas no effect of pesticides was detected at 32 °C (all p values > 0.12) (Fig S5). Body mass loss was affected by temperature only (Table 2), with fish exposed to 32 °C losing more body mass than fish exposed to 22 °C (t = −2.63, P = 0.0093).

3.5. Immunity

Before exposure to pesticides at T0, there were no differences in lysozyme (χ_2 = 3.69, P = 0.59) or peroxidase activity (χ_2 = 3.37, P = 0.64) among groups. Lysozyme variations over the experiment were not affected by treatments (Table 2). In contrast, peroxidase activity variations were affected by both temperature and pesticides and their interaction (Table 2). More precisely, posthoc tests show that 32 °C fish exposed to a low dose of pesticides had a higher increase in peroxidase compared to 22 °C and 32 °C control groups (Fig S6).

3.6. Behavior

Activity at T96 was affected by pesticide exposure and by temperature (Table 2), with fish exposed to a high dose of pesticides being less active than control fish whatever the temperature (estimate = −46.63 ± 16.45, t = 2.83, P = 0.0052), and fish from the 32 °C groups being more active than fish from the 22 °C groups (estimate = 8.34 ± 1.35, t = 6.19, P < 0.001). More specifically, posthoc tests show that at 22 °C, fish exposed to a high dose of pesticides were marginally less active than control fish (W = 439, P = 0.064), whereas at 32 °C, only fish exposed to a low dose of pesticides were significantly less active than control fish (W = 479, P = 0.0038) (Fig. 4A). There was no difference in mean speed, trajectory changes, and exploration between pesticide groups and these behaviours were only affected by temperature (Table 2). More specifically, fish exposed to a high temperature swam faster (estimate = 0.054 ± 0.27, t = 2.00, P = 0.047), changed trajectory more often (estimate = 0.52 ± 0.21, t = 2.42, P = 0.017), and explored more zones compared to fish acclimated to 22 °C (estimate = 0.11 ± 0.040, t = 2.59, P = 0.010).

Feeding behavior (number of pellets eaten) was affected by both temperature and pesticides (Table 2). Fish at 32 °C ate more pellets than 22 °C fish (estimate = 0.029 ± 0.012, P = 0.017) (Fig. 4B). Fish exposed to a high dose of pesticides ate marginally more than control fish (estimate = 0.25 ± 0.14, P = 0.074) (Fig. 4B).

A complementary analysis integrating both the latency to feed and the feeding status (fish eating at least one pellet or no pellet) confirmed that feeding differed among treatment groups (survival analysis: χ^2 = 11.5, P = 0.043): a higher proportion of fish exposed to a high temperature only (C32) and to pesticides (H22, L32 and H32) ate at least one pellet and fed more rapidly than control fish C22 (Supplementary Fig. S7).

4. Discussion

The trend of results obtained in this study indicates that environmentally relevant concentrations of pesticides affected goldfish physiology and behavior, especially at the molecular and cellular levels. Cellular damage induced by pesticides was more severe at 32 °C compared to 22 °C (summary of results in Fig. 5) suggesting high deleterious effects of multiple thermal and toxic stressors.

4.1. Molecular effects of multiple stressors

At the molecular level, DNA damage reflected by micronuclei rate was higher in fish exposed to both pesticides at high dose and high

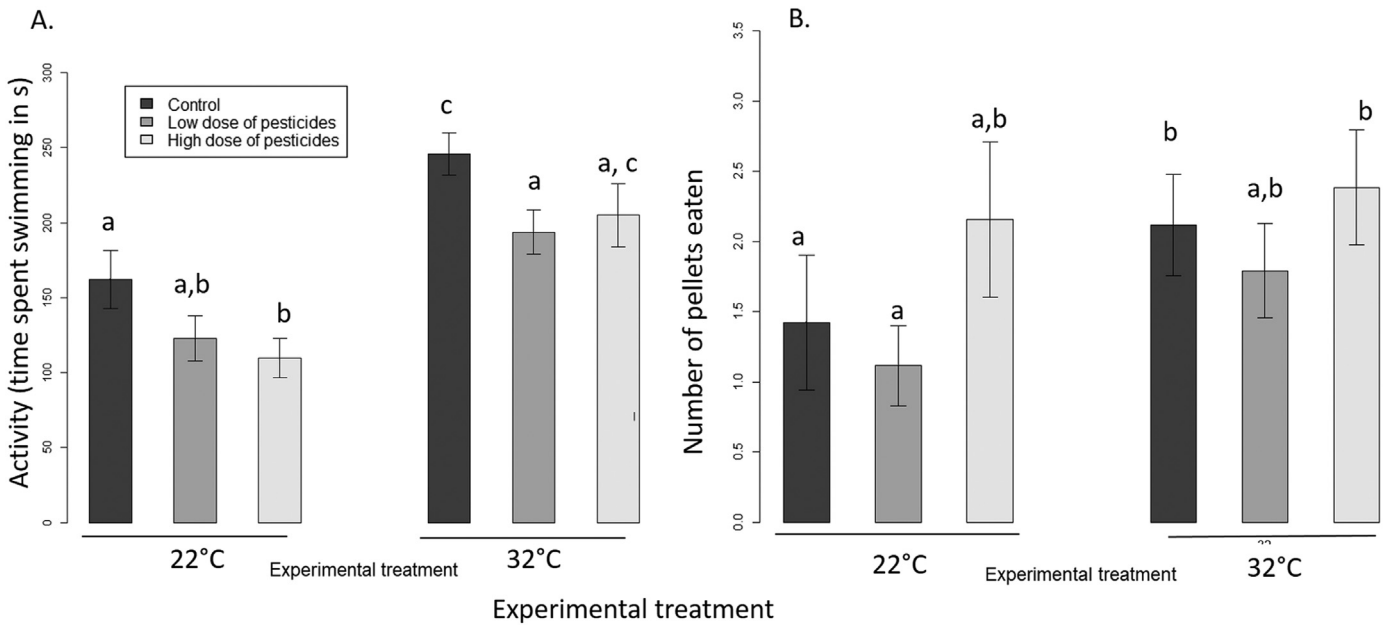

Fig. 4. Routine behaviours, i.e. A. activity (time spent swimming) and B. feeding (number of pellets eaten) of goldfish exposed for 96 h at 22 °C (left) or 32 °C (right) to control water (dark grey), a low dose of pesticides (medium grey), or a high dose of pesticide (light grey) Different letters above bars indicate significant differences between groups.

temperature (H32) compared to control groups. This confirms previous studies showing that pesticides have genotoxic effects and induce DNA alterations in various fish species (Cavalcante et al., 2008; Cavas, 2011; Polard et al., 2011a). Our results further suggest that genotoxic effects of pesticides could be amplified by high temperatures. In general, high temperature itself can increase DNA damage (e.g. Anitha et al., 2000), but Heat Shock Proteins (HSP) and DNA repair mechanisms can often limit such damage depending on the duration and intensity of stressors (Iwama et al., 1998). Accordingly, micronuclei rate did not differ between control fish at 22 °C and 32 °C in our study. We also observed that a combined exposure to thermal and toxic stressors led to important DNA alterations and high micronuclei rate during cell division. This suggests that fish exposed to multiple stressors probably entered in the exhausted phase of the stress and became unable to set up DNA protection and/or repair mechanisms, or that these mechanisms might be not sufficient to repair all damage. However, the underlying mechanisms remain to be explored.

4.2. Histopathological effects of multiple stressors on gills

At the cellular level, several histopathological alterations were also visible in fish exposed to stressors. Increased temperature only had limited effects on gill structure, and mostly resulted in an increase in exchange surface as expected in this species (Sollid et al., 2005). Indeed, goldfish at low temperature have specific interlamellar cell mass (ILCM) between their gill lamellae. This reduces the exchange surface, limits osmoregulation costs and limits the exposure to pathogens and pollutants (Sollid et al., 2005; Sollid and Nilsson, 2006). In addition, goldfish generally show gill remodelling under high temperatures: the ILCM generally decreases at warmer water temperatures (≥ 25 °C),

		Temperature effect	Pesticides effect at 22°C	Pesticides effect at 32°C	Conclusion
Molecules	DNA damage	0	+	++	Stronger effects of pesticides at 32°C
Cells	Gill cellular damage	+	+	++	Stronger effects of pesticides at 32°C
	Liver cellular damage	0	+	++	Stronger effects of pesticides at 32°C
Organs	Gonadosomatic Index (females)	-	-	0	Effects of pesticides at 22°C only
	Hepatosomatic Index	+	0	0	Effect of temperature only
	Body condition Index	+	0	0	Effect of temperature only
Individual	Immune defences	0	0	+	Effects of pesticides at 32°C only (and at low dose)
	Behaviour: activity	+	-	-	Same effect of pesticides at 22°C and 32°C
	feeding	+	0	0	Effect of temperature only

Fig. 5. Summary of the main effects of temperature and pesticides at 22 °C or 32 °C on goldfish across levels of biological organization (0: no effect, -: negative effect, +: positive effect). Taken together, results suggest that pesticides induced more severe molecular and cellular damage on DNA structure, gills and liver cells at 32 °C compared to 22 °C, while other traits were mainly affected by temperature only. This suggests that high temperature aggravates the deleterious non reversible effects of pesticides on cellular and organ structure in a multiple stress context.

thereby increasing the exchange surface area (ESA) (Sollid et al., 2005; Sollid and Nilsson, 2006), as observed in our study at 32 °C (C32 vs. C22 group). This increase in gill exchange area could in turn increase their exposure to pollutants. Accordingly, in our study, more severe gill pathological lesions due to pesticides were observed in fish exposed to a high pesticide dose at 32 °C (H32) compared to fish exposed to the same pesticide dose at 22 °C (H22). Protective mucus that helps preserving the integrity of the gill epithelium (Fernandes and Mazon, 2003) was also reduced in fish exposed to pesticides (H22 and H32), which could participate in the lesions observed.

Several lesions were observed, including lamellar hyperplasia, partial lamellar fusion, lifting of lamellar epithelium and oedema that were very frequent in H32 fish exposed to both stressors, and that can ultimately lead to necrosis. This is consistent with previous studies in goldfish exposed to carbaryl and Polycyclic Aromatic Hydrocarbons (PAHs), which also reported partial lamellar fusion and gill epithelial lifting (Nero et al., 2006; Pfeiffer et al., 1997). Interestingly, in our study, L22 and L32 groups had a similar gill pathological index (I_{Gill}) compared to controls, suggesting that fish could maintain their gill functions at the lowest pesticide concentrations (for 96 h at least), although results should be interpreted with caution given the low sample size (Wolf and Maack, 2017). However, at the higher pesticide concentrations (H22 and H32), the gill pathological index (I_{Gill}) was higher, indicating a higher number and severity of lesions. Fish maintained at 32 °C and exposed to a high concentrations of pesticides (H32) exhibited the highest I_{Gill} (especially a high occurrence of necrosis), indicating irreversible damage that will impair ionic regulation, excretory and respiratory functions of the gills. Taken together, these results indicate a limited impact of single stressors (high temperature or pesticides alone) but a high impact of multiple stressors (pesticides at high temperature) on gill cellular structure and integrity, with potential deleterious consequences for gill function and fish survival.

4.3. Histopathological effects of multiple stressors on the liver

Several histopathological alterations were also visible in the liver of fish exposed to both stressors. The increase of temperature alone had low effects on the hepatic structure of goldfish, while the exposure to pesticides induced a pathology characterized by a fibrosis, melano-macrophage centers and hepatocyte nuclear alterations suggesting an inflammatory syndrome, as well as an accumulation of lipids in hepatocytes. Most fish also showed disorganization of some hepatic areas with an increase in the number of hepatocytes involved in mitosis and a proliferation in blood vessels, which may indicate a carcinogenic process. The pathology was however moderate probably due to the short duration of the exposure (96 h). The pathology described was more frequent and acute in fish exposed to the highest pesticide concentration, which indicated a dose-dependent effect. These results are consistent with previous studies indicating that pesticides can trigger hepatic perturbations (Paris-Palacios et al., 2003, 2000; Paris-Palacios and Biagianti-Risbourg, 2006) and cause disorders of fatty acid metabolism in hepatocytes (Liu et al., 2017). This is also consistent with a previous study reporting proteome disturbances reflecting liver oxidative stress, hepatic steatosis and necrosis in goldfish exposed to the same cocktail of pesticides (Gandar et al., 2017b). The sequestration of lipophilic pollutants and the perturbations of liver metabolism could lead to the increased number of intracellular lipid droplets observed in hepatocytes of H32 fish. This could indicate hepatosteatosis and is consistent with the hepatotoxicity and non-alcoholic “fatty liver” syndrome observed in vertebrates exposed to pesticides (e.g. (Gandar et al., 2017b; Mela et al., 2013; Mesnage et al., 2017; Xing et al., 2012)).

Interestingly, this study shows that the histopathologic perturbation of the liver was more severe when the pesticide exposure was combined to a rise of temperature, suggesting that high temperature aggravates the effects of pesticides on hepatocytes and liver integrity. Although histopathological data need to be analyzed with caution (Wolf and Wheeler, 2018), our results are consistent with a previous study showing different liver proteome profiles in goldfish under multiple stressors compared to a single stressor (Gandar et al., 2017b). This previous study outlined that pesticides at high temperature modified the amount of proteins involved in regulation of oxidative stress, cell cycle and cell death control (Gandar et al., 2017b), which were predicted to cause the observed liver lesions observed in this study.

4.4. Multiple stressors effects at the individual level

At higher levels of biological organization (organ and individual level), gonad mass was reduced in females exposed to a high temperature or pesticides, which is consistent with a reduction in reproductive investment in stressed fish (Matthaei and Lange, 2016; Pankhurst and van der Kraak, 2011; Schreck, 2010). Goldfish exposed to both stressors (L32 and H32) did not have a higher reduction in gonad mass than controls C32, likely because temperature already caused a high reduction of gonad mass. At the individual level, behavioral and immune changes were observed. Activity (as well as speed, trajectory changes and exploration) was increased by temperature as expected by an increase in metabolism and behavioral changes caused by enhanced temperatures (Canal et al., 2015; López-Olmeda and Sánchez-Vázquez, 2011). This is likely due to increased metabolism and higher energy mobilization at high temperature, as expected from previous studies (Gandar et al., 2015). Accordingly, increases in feeding behavior, body condition and HSI were observed in fish exposed to a high temperature.

Interestingly, activity was reduced by pesticides, which is consistent with the hypoactivity syndrome that is often observed in freshwater fish exposed to herbicides (Scott and Sloman, 2004; Shinn et al., 2015), and could explain the reduction in sediment surface reworking observed in goldfish exposed to the same cocktail of pesticides in a previous study (Gandar et al., 2015). However, no other behavioral changes (speed, trajectory, exploration) were observed in response to pesticides. It is not clear yet whether the environmental concentrations chosen have neurotoxic effects and further studies are needed to test neurotoxic effects. However, no decrease in body condition or Hepatosomatic Index were observed under pesticide exposure, which could be explained by the short duration of the exposure (96 h). Alternatively, lipids might have been reallocated from other life-history traits (growth, reproduction) and/or organs (for instance gonads) to maintain liver mass. In addition, pesticides did not trigger a stronger change in behavior under high temperature than under normal temperature, maybe because temperature effects masked the effects of pesticides, or because different metabolic strategies were set up under different temperatures. Further studies specifically testing the allocation of energy in different tissues and functions would be useful to identify the metabolic strategies used by goldfish exposed to multiple stressors.

5. Conclusions

To conclude, a single thermal stress (increased temperature alone) caused behavioral and allocation adjustments (increased activity, feeding and reduced reproductive investment) but did not result in deleterious effects at the cellular level (no deleterious effect on DNA, gills or liver structure). However, high temperature aggravated the effects of pesticides on fish gill and liver. Indeed, pesticides at low temperature (22 °C) had limited deleterious effects on behavior, gills and liver structure, but stronger deleterious effects at 32 °C. More specifically, combined thermal and toxic stressors resulted in high level of irreversible damage of the gills and liver (apoptosis, inflammation, necrosis), with potential consequences for fish osmoregulation, detoxification and survival. This suggests that high water temperatures could enhance the negative effects of pesticides on freshwater fish. Although the chosen model species *Carassius auratus* is a non-native species in Europe, it is now widely found in Europeans rivers and lakes, and has a

higher range of temperature tolerance than several native European fish species. It is thus likely that several other wild freshwater fish species might be severely impacted by combined thermal and pesticide stressors. Given that climate change will likely increase the duration and intensity of heat waves in the next decades (IPCC 2014), this raises concerns about potential synergistic effects of climate change and pollution, and highlights the need for multiple stress approaches to better predict the impacts of human activities on aquatic wildlife.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2019.01.085.

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